Prolyl hydroxylase-1 negatively regulates $I \kappa B$ kinase- β , giving insight into hypoxia-induced NF κB activity

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Hypoxia is a feature of the microenvironment of a growing tumor. The transcription factor NFkB is activated in hypoxia, an event that has significant implications for tumor progression. Here, we demonstrate that hypoxia activates NFkB through a pathway involving activation of $I\kappa B$ kinase- β (IKK β) leading to phosphorylationdependent degradation of $I\kappa B\alpha$ and liberation of $NF\kappa B$. Furthermore, through increasing the pool and/or activation potential of IKK β , hypoxia amplifies cellular sensitivity to stimulation with TNF α . Within its activation loop, IKK β contains an evolutionarily conserved LxxLAP consensus motif for hydroxylation by prolyl hydroxylases (PHDs). Mimicking hypoxia by treatment of cells with siRNA against PHD-1 or PHD-2 or the pan-prolyl hydroxylase inhibitor DMOG results in NFkB activation. Conversely, overexpression of PHD-1 decreases cytokine-stimulated NFkB reporter activity, further suggesting a repressive role for PHD-1 in controlling the activity of NFkB. Hypoxia increases both the expression and activity of IKK β , and site-directed mutagenesis of the proline residue (P191A) of the putative IKK β hydroxylation site results in a loss of hypoxic inducibility. Thus, we hypothesize that hypoxia releases repression of NFkB activity through decreased PHD-dependent hydroxylation of IKK β , an event that may contribute to tumor development and progression through amplification of tumorigenic signaling pathways.

IKK

During cancer progression, a state of hypoxia occurs as the developing tumor outgrows the local blood supply, leading to a drop in pO₂ (1), a condition that should prove rate limiting for tumor growth. However, growth of the tumor is paradoxically facilitated through promotion of a pathway maladaptive for the host involving activation of the hypoxia-specific transcriptional regulator, hypoxia inducible factor-1 (HIF-1), which regulates the expression of genes promoting angiogenesis, vasodilatation, glycolysis, and erythropoesis (2). Furthermore, hypoxia also activates NFκB (3), a transcription factor important in the promotion and progression of tumor development and survival (4). The net result of HIF-1 and NFκB activation by hypoxia is increased tumor oxygenation and survival/growth, respectively. Thus, inhibition of these pathways represents a potentially important therapeutic window of opportunity in the treatment of cancer.

The mechanisms by which HIF-1 is activated in hypoxia are relatively well understood (5). HIF-1 α is constitutively synthesized at a high level in normoxia, but its level is repressed by members of the 2-oxoglutarate-dependent dioxygenase superfamily, namely the prolyl hydroxylases (PHDs). Three PHD isoforms have been described to date (PHD-1, PHD-2, and PHD-3). Oxygen-dependent modification of specific proline residues within consensus LxxLAP motifs (P402 and P564) in HIF-1 α by these enzymes, primarily the PHD-2 isoform, results in the targeting of HIF-1 α for ubiquitination through an E3 ligase complex initiated by the binding of the

Von Hipple Lindau protein (pVHL) and subsequent proteasomal degradation. A further hydroxylation of N803 in the transactivation domain of HIF-1 α by Factor Inhibiting HIF (FIH), an asparagine hydroxylase, represents a second mechanism of oxygen-dependent repression through inhibition of transactivation (6). Similar mechanisms exist for HIF-2 α (5). The hypoxic sensitivity of the HIF pathway is achieved by the absolute requirement of PHD enzymes for molecular oxygen as a cosubstrate. Inhibition of this pathway in hypoxia with the resultant stabilization and transactivation of HIF- α subunits represents a paradigm for oxygen sensing and hypoxia-responsive alterations in gene expression (7).

NFκB-like HIF-1 is a prosurvival transcription factor implicated in tumorigenesis through increasing the expression of genes that inhibit apoptosis and growth arrest in premalignant cells and promote tumor progression through production of cytokines (4). The mechanisms of NFkB activation have been best characterized for their role in inflammation in response to a host of proinflammatory ligands (e.g., TNF α , IL-1, and lipopolysaccharide). A complex sequence of events resulting from receptor occupation by these ligands triggers cascades involving a diverse array of adaptor molecules and enzymes that are ligand-specific (8). There is, however, a convergence point for these signals at the level of the IkB kinases (IKK α , β , and γ) that form the IKK complex (9). Although it is clear that hypoxia activates NFkB, the signaling pathways remain unclear. In the current study, we sought to interrogate the signaling pathways leading to NFkB activation in hypoxia. We investigated whether similar oxygen-sensing mechanisms that exist for HIF-1 may also exist for NFκB. Specifically, we investigated a role for the PHDs.

Results

Hypoxia Activates NFκB. Consistent with previous studies, hypoxia activated NFκB (10, 11). Hypoxia stimulated a 1.9 ± 0.1 - and 5.9 ± 1.7 -fold increase in basal NFκB-dependent transcriptional activity at 24 and 48 h, respectively, as measured by the NFκB-dependent luciferase reporter assay (Fig. 1*A*). Furthermore, this event is preceded by increased levels of nuclear NFκB (as demonstrated by p65 binding to immobilized oligonucleotides containing the con-

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Abbreviations: HIF, hypoxia inducible factor: PHD, prolyl hydroxylase.

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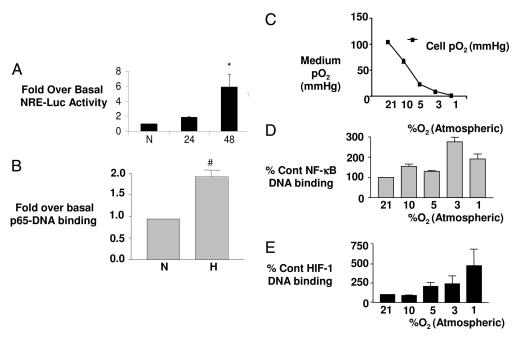


Fig. 1. Hypoxia activates NF κ B. (A) HeLa cells transfected with an NF κ B-luciferase reporter and exposed to 1% O₂ (0–48 h) demonstrate increased NF κ B activity. (B) Nuclear extracts from HeLa cells exposed to preconditioned hypoxic medium (1% O₂; 4 h) demonstrate increased NF κ B DNA binding. (C) HeLa cells exposed to graded hypoxia (21–1% O₂; 24 h) demonstrate decreased extracellular pO₂ values as measured by fluorescence quenching oxymetry and increased NF κ B and HIF-1 α DNA binding activity (D and E).

sensus NF κ B response element) detectable 4 h after exposure to preconditioned hypoxic medium (1.8 \pm 0.29-fold; P < 0.05; 1% O₂; Fig. 1B). Next, cells were exposed to graded hypoxia (21%, 10%, 5%, 3%, and 1% O₂) in separate ambient atmospheric hypoxia chambers. Using oxygen quenching oxymetry, we determined extracellular oxygen tensions at the level of the monolayer to be 105 \pm 2.9, 67 \pm 1.6, 23 \pm 1.0, 9 \pm 1.0, and 2 \pm 0.2 mmHg (1 mmHg = 133 Pa), respectively (Fig. 1C). DNA binding activity of p65 and HIF-1 in nuclear fractions was measured in the same cell lysates and was related to oxymetry readings. Whereas the peak of p65 DNA binding in hypoxia occurred at a cell pO₂ of 9.2 \pm 0.9 mmHg (Fig. 1D), peak HIF-1 DNA binding occurred at a relatively lower pO₂ value (1.7 \pm 0.2 mmHg; Fig. 1E).

TNFα-Induced NFκB Nuclear Binding Is Enhanced in Hypoxia. To determine possible functional consequences for our observations, we examined whether hypoxia increased cellular sensitivity to a stimulus of the NFκB pathway, TNFα. HeLa cells were exposed to a preconditioned hypoxic medium (1% O_2 for 1 h) before treatment with low doses of TNFα (0.01–0.1 ng/ml for 1 h). Nuclear lysates were prepared, and DNA-binding assay was carried out. As predicted, TNFα-dependent activation of the NFκB pathway was significantly enhanced in cells in hypoxia (P < 0.05; Fig. 2).

Hypoxia Activates NF_κ**B Through IKK.** The IKK complex is the convergence point for many diverse NF_κB-activating stimuli including TNF α , LPS, and IL-1 (8). We investigated whether hypoxia-dependent NF_κB activation involves this pathway. Cells were exposed to a preconditioned hypoxic medium (1% O₂), and the phosphorylation status of IKK α / β was determined by Western blot analysis. Hypoxia results in a rapid phosphorylation of the IKK α / β complex detectable after 5 min, which is temporally upstream of IκB α phosphorylation on S32 and S36 (maximal after 15 min) and, in turn, is temporally upstream of IκB α degradation (Fig. 3A). The transient nature of IκB α phosphorylation is consistent with reports of the cyclical nature of NF κ B activation in response to other stimuli

(12). Critically, these measurements were made in the same cell lysates. Thus, hypoxia likely activates NF κ B through IKK-dependent mechanisms. Abolition of HIF-1 expression in HeLa cells using siRNA did not alter hypoxia-induced I κ B α phosphorylation (Fig. 3B), indicating that hypoxia-induced activation of the NF κ B pathway is independent of HIF-1 α .

PHD Inhibition Stimulates NFκB Activity. Prolyl hydroxylases are central to oxygen-sensing pathways leading to HIF-1 α activation as a result of their absolute requirement upon molecular oxygen as a cofactor for enzymatic activity (2). The above data led us to the hypothesis that decreased PHD activity in hypoxia may also underlie NFκB activation. To further test this, we examined components of the NFκB pathway for the presence of the previously described, conserved LxxLAP motif for proline hydroxylation in HIF-1 α (13). Whereas p65, p50, IκB α , and NEMO (IKK γ) are without this sequence, both IKK α and IKK β contain the motif (Fig. 4A). This motif is evolutionarily conserved between humans, mice, and zebrafish. Interestingly, this motif is adjacent to the sites of phosphorylation of IKK β (177/181) on the activation loop, making it likely accessible to modification by enzymes such as PHDs.

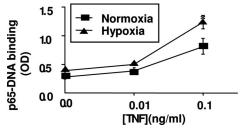


Fig. 2. NFκB DNA-binding assay in HeLa cells exposed to preconditioned hypoxic medium (1% O_2) for 1 h before TNFα treatment (0.01–0.1 ng/ml for an additional hour) demonstrates enhanced NFκB-nuclear binding when compared with normoxic controls.

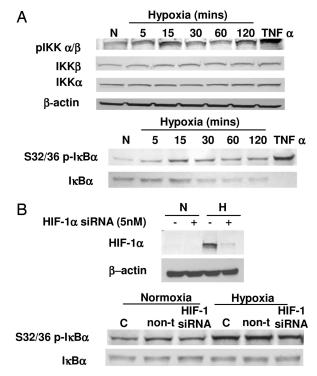


Fig. 3. Hypoxia activates NF_κB through the IKK complex. (*A*) HeLa cells exposed to preconditioned normoxic (N; 21% O₂) or hypoxic (H; 1% O₂) media (5–120 min) demonstrate temporally sequential activation of IKK α / β , phosphorylation of IκB α , and degradation of IκB α . (*B*) HeLa cells transfected with siRNA against HIF-1 α or nontarget siRNA exposed to hypoxia (1% O₂; 24 h) demonstrate effective HIF-1 α knockdown. siRNA against HIF-1 α does not prevent hypoxia-induced phosphorylation of IκB α .

To investigate the possibility that PHDs are playing a role in NFκB activation in hypoxia, we used PHD isoform-specific siRNA (14). Using this approach, we effectively knocked down each of the PHD-1, -2, and -3 isoforms by 73.3%, 80.3%, and 93.6%, respectively. Quantitation of PHD knockdown as measured by quantitative RT-PCR is demonstrated in Fig. 4B. Knockdown of each individual PHD isoform was without effect on expression levels of the other isoforms as measured by quantitative RT-PCR. HeLa cells transfected with an HIF-1dependent luciferase reporter construct demonstrated sensitivity to PHD-2 knockdown as previously reported (ref. 13 and data not shown). Interestingly, cells transfected with an NFκBdependent reporter construct demonstrated the greatest reporter activity in cells deficient in the PHD-1 isoform, indicating that the dominant isoform specificity for PHDs may differ for the HIF-1 and NF κ B pathways (Fig. 4C). Conversely, artificial transient overexpression of PHD-1 in normoxia causes a decrease in TNF α -stimulated NF κ B activity (Fig. 4D).

HeLa cells transfected with an NFκB-luciferase reporter construct were treated in normoxia with the pan-hydroxylase inhibitor DMOG (1 mM) for 24 h. DMOG treatment resulted in a 2.2 ± 0.4 -fold increase in NFκB reporter activity compared with vehicle-treated control cells (P < 0.05, n = 4; Fig. 4E). To assess whether DMOG was acting proximally, at the level of initiation of the NFκB response, we exposed HeLa cells to 1 mM DMOG over a short time course (0–75 min) and demonstrated phosphorylation of S32/36 residues of IκBα, which was maximal after a 30-min exposure (Fig. 4F), thus indicating that PHD inhibition increases IKK activity. Collectively, these data indicate a repressive role for PHD-1 in NFκB signaling. Furthermore, PHD inhibition by DMOG (1 mM, 24 h) or PHD1 siRNA caused a significant increase in the expres-

sion of COX-2, an important NF κ B-dependent inflammatory marker (Fig. 4 G and H).

Hypoxia Alters the Cellular Pool of IKK α and IKK $\beta\mu$. HIF-1 stability is controlled by the PHD-dependent hydroxylation of specific residues in the CODD and NODD regions. The presence of an HIF- α LxxLAP consensus motif in IKK β led us to examine the expression of these proteins under hypoxia. In line with previous studies (15), HeLa cells exposed to graded hypoxia for 24 h demonstrated increased IKK β protein levels, whereas IKK γ /NEMO (which does not contain the hydroxylation motif) did not increase (Fig. 5A). We next investigated the impact of hypoxia on IKK β expression in more physiologically relevant cell lines of immune and epithelial origin. Similar to HeLa cells, IKK β levels were increased by graded hypoxia in THP-1 monocytic cells and in CaCo-2 intestinal epithelial cells in response to instantaneous hypoxia (1% O₂) (Fig. 5B).

Because IKK β has been described as the primary positive regulator of NFκB activity in inflammatory processes and as the molecular link between inflammation and cancer (16–18), we focused our subsequent studies on this isoform. To assess the role of the proline residue within the LxxLAP motif of IKKβ (P191), we used a site-directed mutagenesis strategy to mutate P191 to an alanine residue. HeLa cells were transiently transfected with wild-type IKKβ or P191A IKKβ DNA. Forty-eight hours after transfection, cells were exposed to normoxia or preconditioned hypoxic medium (3% O_2 for 2 h; $pO_2 = 19.9$ mmHg). Cytosolic extracts were prepared, and IKK β expression levels were compared by Western blot analysis. Cells transfected with the wild-type vector demonstrated elevated levels of IKKβ in hypoxia. Cells transfected with the P191A IKKβ vector demonstrated a loss of hypoxic inducibility (Fig. 5C). This is suggestive of a key role for P191 in the hypoxic regulation of IKKβ levels.

Interestingly, digital residue replacement modeling indicates that a modeled IKK peptide containing the LxxLAP motif has the characteristic boomerang structure of the HIF-1 CODD sequence, which is also subject to prolyl hydroxylation by PHDs (19). Furthermore, based on the crystal structure of the HIF-1 CODD peptide interaction with VHL (19), we were able to model an IKK β -pVHL interaction without having to drastically modify the pVHL structure (data not shown).

Coimmunoprecipitation studies demonstrate an interaction between VHL and IKK β in normoxic cells (Fig. 5D); however, this does not appear to result in ubiquitination or proteasomal degradation of IKKβ (data not shown). Furthermore, coimmunoprecipitation studies reveal the interaction between PHD1 and both IKK α and β isoforms. Although this indirectly indicates that IKK is hydroxylated, further critical experimentation using mass spectrometric analysis will be necessary to directly demonstrate hydroxylation. A distinct possibility is that inhibition of IKK activity by hydroxylation of P191 may be due to steric hindrance of the phosphorylation of the nearby residues S177 and S181 within the activation loop. Alternatively, binding of VHL to hydroxylated IKK may prevent activation through the blockage of the serine phosphorylation residues on the activation loop. Thus, we propose that hydroxylation represses IKK β activity by altering protein expression or by preventing its activation through phosphorylation on S177/S181.

Discussion

PHD enzymes are critical in sensing and transducing the HIF-dependent transcriptional response to hypoxia, an event clearly demonstrating an absolute requirement for molecular oxygen as a cosubstrate (7, 20). Thus, PHDs are true oxygen/hypoxia sensors. However, the transcriptional response to hypoxia is not restricted to HIFs with NFkB also demonstrating hypoxic sensitivity and impacting the resultant hypoxic transcriptome (3). However, to date,

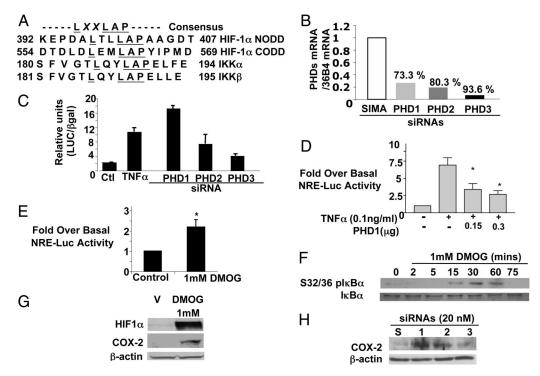


Fig. 4. PHDs suppress NFκB activity. (A) IKK α and IKK β but not NEMO (not shown) contain conserved LxxLAP motifs. (B) HeLa cells transiently transfected with PHD siRNA demonstrate effective isoform-specific knockdown by quantitative RT-PCR. Data are shown from a representative experiment (n=3 in total). (C) HeLa cells were cotransfected with isoform-specific PHD siRNAs (20 nM), the reporter vector (pNFκB-LUC), and 100 ng of a β -galactosidase construct. As a control for NFκB activation, cells were incubated with TNF α (10 ng/ml). Forty-eight hours after transfection, cells were lysed and luciferase and β -galactosidase activity were measured (37). Results are expressed as the fold induction over control. (D) HeLa cells were transiently cotransfected with pEGLN2-FLAG (PHD1; 0.15–0.3 μ g) or empty pcDNA (0.15–0.3 μ g) vector and pNFκB-LUC. After transfection, cells were treated with TNF α (0.1 ng/ml) for 24 h. Whole-cell lysates were prepared, and a luciferase assay was carried out. Results are protein-normalized RLU values expressed as fold over basal luciferase activity. (E) HeLa cells were transiently transfected with an NFκB-promoter reporter construct. After transfection, cells were exposed to DMSO-vehicle (control) or 1 mM DMOG and maintained at 21% O₂ for 24 h. Results shown are protein normalized RLU values. (F) Immunoblotting for anti-phospho-532/36 IκB α and total IκB α was carried out on DMOG-treated HeLa cells (1 mM; 2–75 min). (G) HeLa cells were exposed to DMOG (1 mM, 24 h) or vehicle. Whole-cell extracts were immunoblotted for HIF-1 α , Cox-2, or β -actin. (H) Three PHD isoforms were knocked down in HeLa cells as described above, and Cox-2 expression was determined by Western blot analysis.

our understanding of PHD involvement in hypoxic signaling has been restricted mainly to the HIF-1 pathway. Because of its role in tumor progression, NFκB has important pathophysiologic implications in cancer, a condition involving significant tissue hypoxia.

Hypoxia-activated NFκB is demonstrable by the nuclear accumulation of the p65 subunit as well as by reporter gene assay and correlates with decreased cellular pO₂. Furthermore, hypoxiainduced NFkB activity is preceded by temporally sequential IKK activation, IkB phosphorylation, and IkB degradation, indicating that hypoxia activates NFkB through increased IKK activity. The IKK-dependence of hypoxia-induced NFκB activity is consistent with most other stimuli of this pathway. Although previous studies have hypothesized roles for tyrosine phosphorylation (21), reactive oxygen species generation (22), and p42/44 or PI-3-kinase (23) in mediating NFκB responses in hypoxia, none of these theories clearly demonstrates a direct relationship between decreased molecular oxygen and NFκB activation such as through altered PHD activity. Our model proposes that while hypoxia activates NFkB it also increases the sensitivity of the NFkB pathway to activation through proinflammatory stimuli, such as cytokines. Thus, the importance of this pathway may be greatest where hypoxia occurs against the backdrop of increased inflammatory activity [such as in a growing tumor (24)] where amplification of a stimulated response may heavily affect tumor-promoting gene expression.

Alternative signaling targets for proline hydroxylation in the transcriptional response to hypoxia are attractive in developing the theory that hydroxylating enzymes are central to cellular oxygen sensing (7, 20). In the current study, we demonstrate that $IKK\beta$, a critical regulator of $NF\kappa B$ activity, contains a conserved

prolyl hydroxylation consensus site homologous with the two conserved sites within the oxygen-dependent degradation domain (ODD) of HIF-1 (LxxLAP). Like HIF-1 α , the pool of IKK β in cells is increased in hypoxia; however, unlike HIF α , the absolute expression of IKKs is not determined by the presence of oxygen because basal expression is clearly detectable. Thus, it appears that hypoxia is more a modulator of IKK expression and activation than an absolute determinant. This may be due to the presence of just a single LxxLAP motif in IKK β rather than the two present in HIF-1 α . A recent study has demonstrated the requirement of a leucine residue within 10 residues of the LxxLAP motif for hydroxylation of the consensus motif (25). Two leucine residues reside closely downstream of the LxxLAP motif in IKK\(\beta\). Importantly, the putative site of hydroxylation (P191) on IKK β resides just 10 residues from the known phosphorylation site S181, which is required for IKK β activation.

S181 is positioned in the vicinity of P191 in the well conserved protein kinase activation loop, and P191 itself is predicted to be positioned in the substrate binding cleft. Experimental studies (26) of a prototypic protein kinase (the cAMP-dependent protein kinase, PKA) show that the conformation and dynamics of the activation loop are known to be linked with the phosphorylation of S181. Thus, it is possible that hydroxylation of P191 alters the conformation of the activation loop, making phosphorylation difficult or ineffective (i.e., the kinase might be phosphorylated but inactive) and/or impairs the binding of the protein kinase substrate. This would cause a decrease in IKK β (and subsequently NF κ B) activity. Similarly, it is also conceivable that phosphorylation renders hydroxylation more difficult

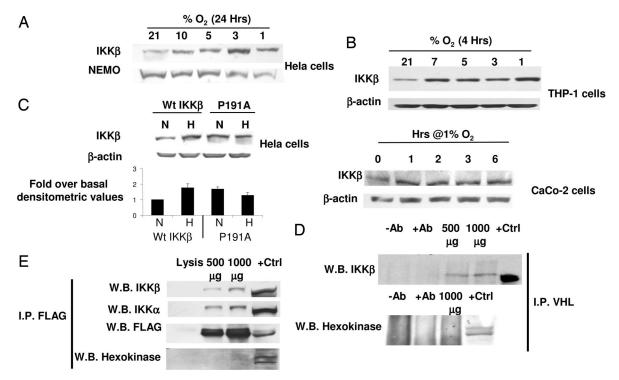


Fig. 5. The cellular pool of IKK β is increased in hypoxia. HeLa (A) and THP-1 and CaCo-2 (B) cells were exposed to graded hypoxia (21–1% O₂) for 24 h or instantaneous hypoxia (1% O₂) for 0–6 h. Whole-cell extracts were immunoblotted for IKK β , NEMO, and β -actin where indicated. (C) HeLa cells were transiently transfected with 1 μ g of wild-type IKK β or P191A mutant IKK β . Forty-eight hours after transfection, the cells were maintained in normoxia (N; 21% O₂) or exposed to preconditioned hypoxic medium (H; 3% O₂) for 2 h. Cytosolic extracts were immunoblotted for IKK β and β -actin. Alterations in protein expression were measured semiquantitatively by using densitometric analysis (n=4). (D) pVHL was immunoprecipitated from whole HeLa cell extracts (500 or 1,000 μ g of total protein), and immunoprecipitates were immunoblotted for IKK β and hexokinase (similarly sized negative control). (E) NETN lysates from HeLa cells transiently overexpressing PHD-1-FLAG were immunoprecipitated by using a specific anti-FLAG resin. Immunoprecipitates were immunoblotted for IKK α , FLAG, and hexokinase.

because the hydroxylation site in active $IKK\beta$ could be masked by the substrate. Additionally, the activation loop would be less dynamic and hence less accessible to PHDs.

The role of IKK β in oncogenesis and inflammation is well established, but there is emerging evidence that IKK α may have antiinflammatory functions (27, 28). We also observed an increase in IKK α in hypoxia (data not shown). The observation that both IKK α and IKK β are hypoxia-sensitive, albeit differentially, suggests the possibility of a counterbalancing mechanism where a p65-mediated response could be resolved over a course of hypoxia.

Genetic or pharmacologic interference with PHD activity results in activation of NFkB-dependent signaling in normoxia. Mutation of the hydroxylated proline residues of HIF-1 α results in a loss of hypoxic inducibility (29). Similarly, mutation of proline residue 191 in IKK β , which resides within the consensus sequence, results in a similar effect. Although sensitive to knockdown of both PHD-1 and PHD-2, NF κ B activation appears to be more sensitive to silencing of the PHD-1 isoform, suggesting somewhat differential regulation to HIF-1, which is predominantly regulated by PHD-2. These data suggest the intriguing possibility that hypoxia increases IKK stability through an inhibition of protein hydroxylation, resulting in evasion of degradation in a similar manner to HIF-1. Indeed, it is worth noting that a number of studies have demonstrated that the E3 pVHL suppresses NFκB activity in normoxia (30–32). However, although IKK β interacts with VHL in normoxia, we found no evidence for its ubiquitination/degradation. Furthermore, the rapid kinetics of IKK β activation in hypoxia suggests the likelihood that the primary repressive role of P191 hydroxylation would be through inhibition of phosphorylation of S177/S181. This may occur through VHL masking of the activation loop.

Thus, hypoxia increases the expression and activation of IKK, leading to increased sensitivity of cells to inflammatory stimuli

such as cytokines. This has important implications in cancer where elevated levels of proinflammatory cytokines coexist with hypoxia in the microenvironment of the tumor (33). Furthermore, this is consistent with previous studies demonstrating synergy between hypoxia and inflammatory cytokines in the activation of cells (10, 34).

In summary, we propose that hypoxia, through inhibiting PHD activity, increases the activity of cellular IKK β , an event that amplifies the cellular capacity for response to cytokines. Furthermore, we believe that such events may directly impact on tumor development through enhanced expression of genes that promote tumor development and growth.

Methods

Cell Culture and Hypoxia. HeLa, CaCo-2, and THP-1 cells were placed in one of four hypoxia chambers (Coy Laboratories, Grass Lake, MI; Ruskin Technologies, Leeds, U.K.) allowing the establishment of graded, humidified, ambient, atmospheric hypoxia of 21%, 10%, 5%, 3%, and 1% O_2 with 5% CO_2 and a balance of N_2 in all cases. Extracellular pO₂ measurements were made by using fluorescence quenching oxymetry (Oxylite-2000; Oxford Optronix, Oxford, U.K.). Hypoxia did not induce apoptosis or necrosis (data not shown). Temperature was maintained at 37° C. Where outlined, instantaneous hypoxia was achieved by exposure of cells in hypoxia chambers to preconditioned media.

Western Blot Analysis. Cytoplasmic or whole-cell lysates were separated by SDS/PAGE, transferred to nitrocellulose membranes, and immunoblotted as described (35). IKK α , IKK β , and phospho-IKK α (S180)/IKK β (S181) polyclonal antibodies (Cell Signaling), IKK γ /NEMO polyclonal antibody (Santa Cruz Biotechnology), I κ B α polyclonal antibody (Upstate Biotechnology), FLAG poly-

clonal antibody (Sigma), Hexokinase polyclonal antibody (Biogenesis), Cox-2 polyclonal antibody (Cayman Chemical), phospho- $I\kappa B\alpha$ monoclonal antibody (Cell Signaling), and HIF-1 α monoclonal antibody (BD Transduction Laboratories) were used.

NFkB-DNA Binding Assays. Nuclear lysates for DNA-binding assays were prepared as per the manufacturer's instructions (TransAm kit; ActiveMotif). The nuclear extract was incubated with an immobilized oligonucleotide on a 96-well plate (containing a specific transcription factor binding site as indicated) and detected by the transcription factor ELISA.

Generation of IKK β Mutant by Site-Directed Mutagenesis. The P191A IKKβ mutant was generated by using a site-directed mutagenesis kit described previously (36). Plasmid constructs were verified by using DNA sequencing. Primers designed for IKKβ were as follows: forward, 5'-CCCTGCAGTACCTGGC-CGCAGAGCTACTGGAGC-3'; reverse, 5'-GCTCCAGTAG-CTCTGCGGCCAGGTACTGCAGGG-3'.

Transient Transfection. Transient transfection of plasmid DNA was performed in HeLa cells (50-70% confluent) by using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. After transfection, cells were exposed overnight at 37°C (5% CO₂). Medium was replaced, and cells were exposed to experimental treatment as described.

Reporter Assay. Cells were transfected with an NF κ B-luciferase construct (Stratagene Cis-Reporting Systems) with a synthetic promoter containing a concatomer of five NFkB response elements. Cell lysates were prepared by using a 1× luciferase lysis buffer (Promega). Substrate was added to the cell lysate, and luciferase activity was measured in a luminometer. Experiments were carried out in duplicate or triplicate, and luciferase values were normalized to cotransfected CMV Renilla luciferase, β -Gal, or protein control values.

RNA Interference by siRNA. HeLa cells were grown to 40-50%confluency and transfected with 5 nM specific siRNA against HIF- 1α or control nontarget siRNA (Dharmacon) by using a Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions. Cells were maintained in antibiotic-free media for 48 h after transfection to achieve maximal knockdown of the target gene. RNA interference by siRNA against PHD isoforms was carried out as described (14) and quantified by quantitative RT-PCR (Applied Biosystems).

Coimmunoprecipitation Studies. Whole HeLa cell lysates were prepared in a lysis buffer as described above. Lysate was precleared with 50 µl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) and rotated for 1 h at 4°C. Five hundred micrograms and 1,000 μ g of lysate were incubated with 2 μ g of an anti-VHL antibody (BD Pharmingen) and brought to a final volume of 650 μ l with lysis buffer. The lysis buffer (650 μ l) \pm anti-VHL antibody as well as the nonimmunoprecipitated wholecell extracts were included as controls (+Ab, -Ab, and +Ctrl, respectively). Samples were rotated for 2 h at room temperature. Thirty microliters of protein A/G PLUS agarose beads was added to the samples and controls and rotated for 1 h at 4°C. Samples were centrifuged at 17,968 \times g for 6 min at 4°C. Beads were washed three times in lysis buffer. The supernatant was discarded, and the pellet was resuspended in reducing sample buffer (65 μ l) and boiled for 10 min before electrophoresis.

HeLa cells were transiently transfected with pEGLN2-FLAG. Whole-cell lysates were prepared in NETN buffer [20 mM Tris·HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, protease inhibitor mixture]. Five hundred micrograms and 1,000 μ g of protein lysate were incubated with 30 μ l of anti-FLAG agarose affinity gel (Sigma) and brought to a final volume of 500 µl with an NETN buffer. NETN buffer alone (Lysis) was incubated with the beads as a control, in addition to a nonimmunoprecipitated NETN lysate (+Ctrl). Samples were rotated overnight at 4°C. Samples were centrifuged at 17,968 \times g for 6 min at 4°C. Beads were washed three times in NETN buffer. The supernatant was discarded, and the pellet was resuspended in reducing sample buffer (65 μ l) and boiled for 10 min before electrophoresis.

Statistical Analysis. All data are presented as mean \pm SEM for n independent experiments. Statistical significance was evaluated by using one-way ANOVA or Student's t test for unpaired or paired data where an asterisk corresponds to P < 0.05.

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